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14. ABSTRACT

The purpose of this project is to identify initial biomarker patterns in SLE nephritis using screening proteomic profiling, and advanced proteomic profiling. Subject recruitment has been completed (150 children with SLE, out of which 75 have lupus nephritis). Utility of one of the biomarkers (NGAL) in predicting worsening of global and renal SLE disease activity has been validated. We found 2 proteins significantly over-expressed in Class IV vs Class V lupus nephritis by 2D gel electrophoresis: albumin fragments (25kDa) and α-1-B glycoprotein (60kDa). We found additional proteins differentially expressed in Class IV vs Class V lupus nephritis by SELDI-TOF-MS. Additional proteomic profiling studies using NMR- and MS-based metabonomics have been completed, and LC/MS based protein profiling using Thermo LTQ FT-ICR have been initiated. Overall, these studies will identify a subset of non-invasive biomarkers that identify lupus nephritis sub-classes, and predict the clinical course of the disease. The significance of such biomarkers is that they will provide novel non-invasive tools to identify patients with lupus nephritis, to risk-stratify the subjects for therapies, and to follow the efficacy of therapies.

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Lupus nephritis, Biomarkers, Proteomics

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INTRODUCTION AND SCOPE OF WORK

We propose to identify biomarker patterns in SLE nephritis by pursuing the following specific aims:

Specific Aim 1: Screening proteomic profiling: Initial high-throughput screening proteomic analysis will be done in the Devarajan Lab using 2D gel electrophoresis and Surface-Enhanced Laser Desorption/ Ionization Time-of-Flight mass spectrometry (SELDI-TOF-MS). Changes in proteomic profiles will be confirmed and enhanced using NMR- and MS-based metabonomics, by Dr. Michael Kennedy, Miami University. Changes in proteomic profiles will be compared to changes in currently available renal biomarkers (urinalysis, blood and urine chemistry), medications and other clinical outcomes (overall disease activity, renal and overall damage).

Specific Aim 2: Advanced proteomic profiling: Advanced proteomic studies on selected sample sets will be performed at Applied Biotechnology Branch, Air Force Research Lab, Wright-Patterson Air Force Base (AFRL/HEPB), where LC/MS based protein profiling using Thermo LTQ FT-ICR will provide ultra-high resolution/mass accuracy protein identification, using the LTQ FT-ICR hybrid instrument (Thermo Electron North America LLC). The data will be analyzed by using Bioworks 3.2 software for protein identification along with statistical calculations for protein/peptide probabilities.

BODY

Research Accomplishments for Task 1:
To identify initial biomarker patterns in SLE nephritis using screening proteomic profiling

1.1: Subject Recruitment

All subjects with systemic lupus erythematosus (SLE) targeted for this study have now been recruited. We have recruited approximately 150 children with SLE, including some with and some without active renal disease. We achieved our goal of recruiting 75 patients with active renal disease, 75 patients without active lupus nephritis (LN). Seventy five children with Juvenile Idiopathic Arthritis (JIA, disease controls) and 75 normal siblings of children with JIA (healthy controls) have been recruited. All subjects had at least six study visits to date, and the majority have completed all 7 study visits. Additionally, we have recruited 10 children with Focal Segmental Glomerulosclerosis (FSGS) to serve as a disease control group to better dissect mechanisms of inflammatory lupus nephritis from non inflammatory nephropathies with similar urinary findings.

1.2: Validation of NGAL as a biomarker for predicting SLE disease activity

During the first year of this study, we identified a panel of urinary biomarkers that correlated with SLE renal disease activity. Of the biomarkers, neutrophil gelatinase-associated lipocalin (NGAL) appeared to be the most promising. During this (second) year, we validated the utility of NGAL in predicting impending worsening of global and renal SLE disease activity. A total of 111 patients with SLE were enrolled in a longitudinal, prospective study with quarterly study visits and had at least three study visits. At each visit, global disease activity was measured using three external standards: numerically converted BILAG index, SLEDAI-2K and physician assessment score. Renal and extra-renal disease activity was measured by the respective domain scores. The disease course over time was categorized at the most recent visit (persistently active, persistently inactive, improved or worsening). Plasma and urinary NGAL levels were measured by ELISA, and urinary NGAL was standardized to urinary creatinine. The longitudinal changes in NGAL levels were compared to the changes in SLE disease activity using mixed effects models. Significant increases in standardized urinary NGAL levels of up to 104% were detected up to three months before worsening of lupus nephritis (as measured by all three external standards). Plasma NGAL levels increased significantly by as much as 20% up to three months before worsening of global SLE disease activity as measured by all three external standards. Plasma NGAL levels increased significantly by 26% as early as three months prior to worsening of lupus nephritis as measured by the renal BILAG domain score. We then assessed the diagnostic accuracy of NGAL as a predictive biomarker of the course of SLE nephritis. As shown below in the Receiver operating characteristic curves in Figure 1, urine NGAL predicted probability of worsening kidney function as assessed either by the SLEDAI-2K renal score (panel A) or the BILAG renal score (panel B), with an area under the curve in the 0.80 range. Corresponding sensitivities and specificities at a n optimal cut-off are also shown in Figure 1. We concluded that serial measurement of urinary and plasma NGAL levels are valuable in predicting impending worsening of global and renal SLE disease activity. A manuscript describing these results has been published in ***Arthritis and Rheumatism.*** The manuscript was attached to last year's annual report.

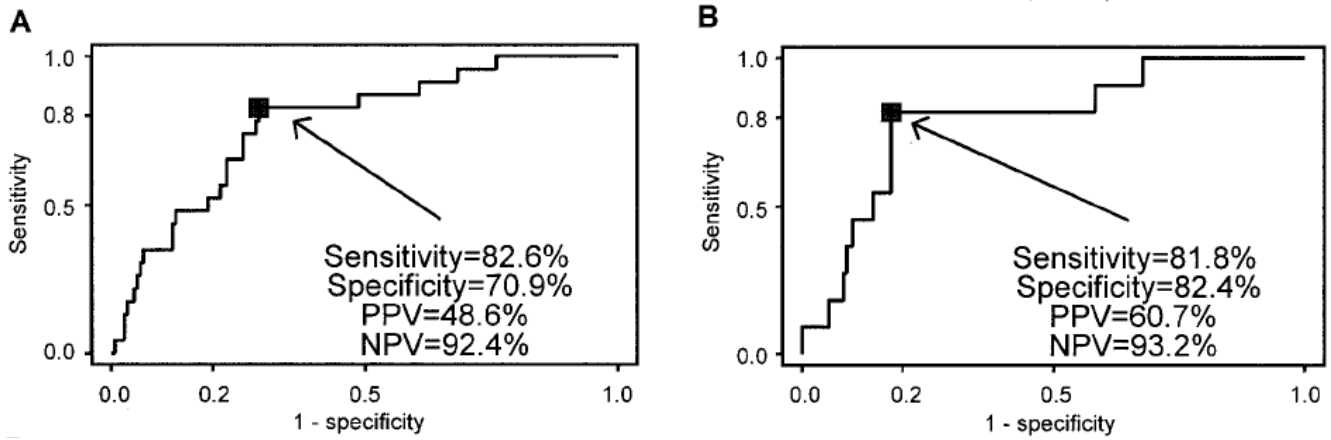

Figure 1. ROC curves for urine NGAL to predict worsening kidney disease using the (A) SLEDAI-2K renal score or (B) BILAG renal score

1.3: Identification of urinary biomarkers for distinguishing subjects with Class IV from Class V lupus nephritis using 2DGE and SELDI-TOF-MS

The ISN/RPS class IV and V Lupus nephritis (LN) show different histological features and differ in prognosis. We aimed to identify non-invasive biomarkers which differentiate between class IV and V LN. Urine samples from 6 children with class IV LN, 7 with class V LN, and 4 with FSGS (disease control) were studied. All samples were collected within 60 days of a kidney biopsy. Two complementary proteomic methods were employed: 2 dimensional gel electrophoresis (2DGE) and SELDI-TOF-MS. We found 2 proteins significantly over-expressed in class IV vs. class V by 2DGE. MALDI-TOF-MS/MS analysis identified these proteins as human serum albumin fragments (25kDa) and α-1-B glycoprotein (60kDa). In SELDI-TOF-MS, we used four different types of ProteinChips and analyzed the spectra with ProteinChip Data Manager 3.07. Identification of the most robustly differentially expressed peaks is being completed. We have identified α-1 antitrypsin (A1AT) as one of the peaks. This protein was significantly ($p < 0.01$; AUC 0.90) over-expressed in Class V vs Class IV LN. The combination of α-1-B glycoprotein and α-1 antitrypsin define part of a signature of urinary biomarkers that clearly distinguish between class IV and class V LN. These findings have important implications not only for biomarker discovery, but also for differential pathogenic mechanisms for LN subclasses.

1.4: Urinary Metabonomic studies in Lupus Nephritis

Class IV and V Lupus nephritis (LN) show different histological features and differ in prognosis. We aimed to identify non-invasive metabonomic biomarkers which differentiate between class IV and V LN. Urine samples from 6 children with class IV LN and 7 with class V LN were studied. All samples were collected within 60 days of a kidney biopsy. Urinary profiling was performed using NMR- and MS-based metabonomics at Miami University, in the laboratory of Dr. Michael Kennedy. Initial spectra and profiles obtained show significant differences between patients with Class IV versus Class V SLE nephritis. Comparison of nuclear magnetic resonance (NMR) spectroscopy-based metabolic profiles of urine samples from Class IV and Class V lupus nephritis patients by principal component analysis (PCA) and visualization showed changes in citrate and taurine (Figures 2 & 3). Class V patients display decreased levels of citrate and increased levels of taurine when compared to Class IV patients.

Changes in concentration of urinary citrate and urinary taurine has previously been linked to renal tubular dysfunction. Urinary citrate excretion is regulated by tubular citrate reabsorption. Cytosolic citrate metabolism, through the enzyme ATP citrate lyase, decreases urinary citrate excretion in metabolic acidosis. Renal tubular acidosis (RTA), a disorder in which the kidney tubules cannot adequately remove acids from the blood to excrete them in the urine, has previously been associated with systemic lupus erythematosus. Citrate helps to solubilize urinary calcium and exhibits reduced excretion in cases of distal tubular RTA. The increase in tubular reabsorption is due to intracellular acidosis because citrate excretion appears to be controlled by the pH of the renal tubular cell. The urinary analysis of citrate from individuals with Class V lupus nephritis provides evidence that these patients have more advanced impairment of renal tubular absorption compared to Class IV patients. In some cases, RTA accompanied by lupus nephritis has been resistant to treatment with corticosteroids because of the permanent damage done to the tubular interstitial tissue. These findings indicate that treatment of Class V lupus nephritis may need to be altered if associated with distal RTA.

The total body pool of taurine is regulated by the kidneys by altering tubular reabsorption. Taurine is the most profuse intracellular amino acid in the human body and is not integrated into proteins. The major physiological role of taurine is cell membrane protection either by acting as an osmoregulator or reducing toxic substances. The amount of taurine excreted can be influenced by renal function and conditions inducing taurine release from cells, such as disease or tissue damage. Because taurine is filtered through the glomerulus and partially reabsorbed in the tubules, extensive damage to the glomuleri as seen in Class V patients may cause taurine leakage from damaged tissue and lead to increased urinary taurine. Elevated levels of urinary taurine may help to diagnose tissue damage localized to the kidneys of lupus nephritis patients and reduce the need for biopsies to distinguish the classifications of kidney disorders.

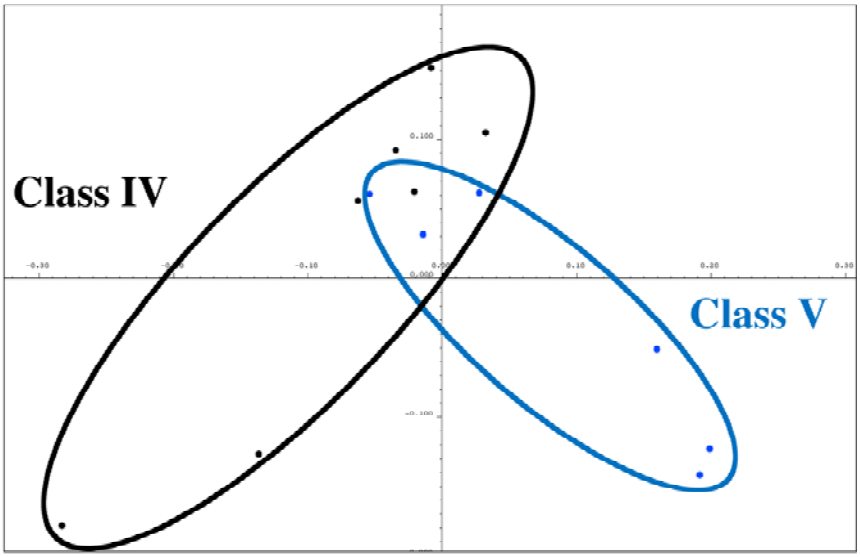

Figure 2. PCA scores plot analysis of NMR data collected from urine samples of Class IV and Class V lupus nephritis patients. Each point in the scores plot represents a NMR spectrum of urine from an individual patient. The ovals encircle all points belonging to either the Class IV group (black) or the class V group (blue). The separation of the ovals indicates distinct metabolic profiles for each group.

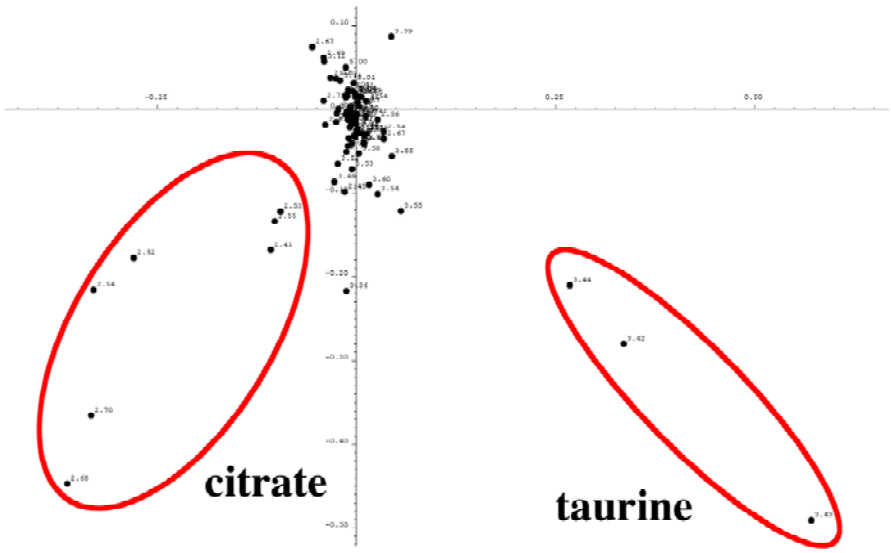

Figure 3. PCA loadings plot analysis of NMR data collected from urine samples of Class IV and Class V lupus nephritis patients. Each point in the loadings plot represents a chemical shift bucket corresponding to a specific metabolite resonance in the NMR spectra of urine from lupus nephritis patients. The ovals encircle all points belonging to either the citrate or taurine metabolites. Points located in the same regions as the scores plot are more abundant in that group, e.g. taurine is more abundant in the Class V group.

Research Accomplishments for Task 2:

To identify biomarkers predictive of SLE nephritis using advanced proteomic profiling:

2.1: Advanced Proteomics in Lupus Nephritis

This has been initiated at the Applied Biotechnology Branch, Air Force Research Lab, Wright-Patterson Air Force Base (AFRL/HEPB), under the direction of Dr. Schlager. LC/MS based protein profiling of urine from SLE patients using Thermo LTQ FT-ICR will provide ultra-high resolution/mass accuracy protein identification. These studies are expected to be completed in the upcoming year. We have requested and have obtained a No Cost Extension to complete these studies.

Preliminary LC-MS/MS2 uncovered the most differences between the groups. Among proteins upregulated in Class V LN were apolipoprotein D, lipocalin-like prostaglandin D synthetase, ITIH4, Caspase 10, uromodulin and CD14. Those most upregulated in Class IV LN were vitamin D binding protein, ceruloplasmin, hemopexin, A1BG and orosomucoid. A1AT has been linked to SLE flares and hemopexin is associated with glomerular disease. It will be important in the upcoming year to confirm these preliminary findings using additional samples from patients with Class IV and Calss V lupus nephritis.

KEY RESEARCH ACCOMPLISHMENTS

- Completion of subject recruitment
- Validation of NGAL as a predictive urinary biomarker for impending worsening of SLE disease activity
- Identification of a urinary biomarker signature that distinguish between class IV and class V LN, that includes the following differentially expressed biomarkers:
    - albumin fragments (25kDa) and α-1-B glycoprotein (60kDa) by 2D gel electrophoresis
    - α-1-antitrypsin by SELDI-TOF-MS
    - citrate and taurine by NMR spectroscopy-based metabolomic profiling
    - apolipoprotein D, lipocalin-like prostaglandin D synthetase, ITIH4, Caspase 10, uromodulin, CD14, vitamin D binding protein, ceruloplasmin, hemopexin, A1BG and orosomucoid by LC-MS/MS2

REPORTABLE OUTCOMES

This section contains information on abstracts submitted (which represent journal manuscripts in preparation).

ABSTRACTS SUBMITTED:
Michael R. Bennett, PhD, Michiko Suzuki, MD, PhD, Shannen Nelson, Josh Pendl, Michael Kennedy, Pavel Shyianov, and Hermine Brunner, MD, Prasad Devarajan, MD. Urinary biomarkers to distinguish Class IV vs Class V lupus nephritis. Abstract submitted to the Annual Meeting of the American Society of Nephrology, 2010, and to the American College of Rheumatology Annual Meeting, 2010. and for poster presentation at the American Society of Nephrology meeting.

CONCLUSION

Thus far, we have completed Task 1 and a portion of Task 2. We have completed subject recruitment, validated one of the biomarkers (NGAL) as a predictive urinary biomarker for impending worsening of SLE disease activity, and identified a urinary biomarker signature that distinguish between class IV and class V LN. This includes albumin fragments (25kDa) and α-1-B glycoprotein (60kDa) identified by 2D gel electrophoresis, α-1-antitrypsin by SELDI-TOF-MS, citrate and taurine by NMR spectroscopy-based metabolomic profiling, and apolipoprotein D, lipocalin-like prostaglandin D synthetase, ITIH4, Caspase 10, uromodulin, CD14, vitamin D binding protein, ceruloplasmin, hemopexin, A1BG and orosomucoid by LC-MS/MS2

Additional proteomic profiling studies using LC/MS based protein profiling using Thermo LTQ FT-ICR have been initiated, and will be completed in the up coming year. These were not able to be completed during the past year, due to several technical and equipment-related difficulties encountered by the Wright Patterson Air Force Base laboratories.

Overall, these studies will identify a subset of non-invasive biomarkers that identify lupus nephritis sub-classes, and predict the clinical course of the disease. The significance of such biomarkers is that they will provide novel non-invasive tools to identify patients with lupus nephritis, to risk-stratify the subjects for therapies, and to follow the efficacy of therapies.

The following work is planned to be completed during the next reporting period:

1. Completion of advanced urinary proteomic profiling of lupus subjects with Thermo LTQ FT-ICR, and identification and characterization of proteins differentially expressed in lupus sub-classes, as revealed by this advanced proteomic technique

Although the work has progressed very well, we must remain cognizant of potential problems in the future, and we must be prepared to address these problems, as summarized below:

(a) Current problems that may impede performance

- Initial SELDI-TOF-MS studies have revealed more than 30 proteins that are differentially expressed in Class IV versus Class V lupus nephritis. This number is larger than initially anticipated. Thus far, we have been able tro identify and validate only one of these candidates, namely α-1-antitrypsin. We will need to perform additional SELDI-TOF-MS experiments on additional samples, in order to establish whether the observed large differences are consistent in a new set of samples.
- Initial 2 dimensional gel electrophoresis (2DGE) and SELDI-TOF-MS have identified only 2 proteins significantly over-expressed in class IV vs. class V: human serum albumin fragments (25kDa) and α-1-B glycoprotein (60kDa). This number is smaller than initially anticipated.
- Initial NMR spectroscopy-based metabolomic profiling have identified only 2 species differentially expressed in class IV vs. class V: citrate and taurine. This number is smaller than initially anticipated.

(b) Anticipated problems

- Initial LC/MS based protein prof iling of urine from SLE patient s using Therm o LTQ FT-ICR has proven to be m ore expensive and associated w ith more te chnical dif ficulties than initia lly anticipated. W e have worked with our collaborators at the Applied Biotechnology Branch, Air Force Research Lab, Wright-Patterson Air Force Base, to accomplish as much of this aim as possible within the initial budget provided, a nd will continue to work with th em during the period of no cost extension that has been granted for the completion of this project.

APPENDIX

Abstract submitted to American College of Rheumatology Annual Meeting, and to the American Society of Nephrology Meeting, 2010

Title: Urinary biomarkers to distinguish Class IV vs Class V lupus nephritis.

Michael R. Bennett, PhD*[1], Michiko Suzuki, MD, PhD[1], Shannen Nelson[1], Josh Pendl, Michael Kennedy, Pavel Shyianov, and Hermine Brunner, MD[1], Prasad Devarajan, MD[1] [1]Cincinnati Children's.

Body: Up to 80% of children with systemic lupus erythematosus have lupus nephritis (LN). The ISN/RPS Morphologic Classification of LN reports on histological features that differentiate between various forms of LN, such as Diffuse Proliferative Class IV and Membranous Class V lesions. Kidney biopsies are the choice for diagnosis of LN, but are impractical to accurately assess the course of LN in clinical practice.

We set out to discover non-invasive urinary biomarkers that can discriminate LN subtypes.

We used 2-dimensional gel electrophoresis (2-DGE), NMR-based metabonomics, surface-enhanced laser desorption/ionization time of flight MS (SELDI), and liquid chromatography tandem mass spectrometry (LC MS/MS2) to investigate novel biomarkers that could distinguish Class IV vs. Class V LN. Urine samples from children with Class IV LN (n=6) and (pure) Class V LN (n=7) collected within 60 days of a kidney biopsy and those of controls with focal segmental glomerulosclerosis (n=4) were tested. Samples were normalized for total protein (2-DGE and LC-MS/MS2) or urine creatinine (NMR and SELDI). Using 2-DGE and MALDI-TOF-MS/MS, we found serum albumin fragments (25kDa) and α1-B glycoprotein (A1BG, 60kDa) significantly over-expressed in class IV vs. class V LN. Using SELDI, we identified Alpha-1 Antitrypsin (A1AT). This protein was significantly ($p < 0.01$; AUC 0.90) over-expressed in Class V vs Class IV LN and FSGS controls. Principal component analysis of NMR metabonomics spectra suggests decreased levels of citrate and increased levels of taurine in Class V when compared to Class IV patients, while LC-MS/MS2 uncovered the most differences between the groups. Among proteins upregulated in Class V LN were apolipoprotein D, lipocalin-like prostaglandin D synthetase, ITIH4, Caspase 10, uromodulin and CD14. Those most upregulated in Class IV LN were vitamin D binding protein, ceruloplasmin, hemopexin, A1BG and orosomucoid. A1AT has been linked to SLE flares and hemopexin is associated with glomerular disease.

The discovery of non-invasive biomarkers that can distinguish LN subtypes would greatly aid in diagnosing and monitoring treatment in LN.